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Redistribution of Alpha-granules and Their Contents in Thrombin-stimulated Platelets

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ABSTRACT The redistribution of β -thromboglobulin (β TG), platelet Factor 4 (PF4), and fibrinogen from the alpha granules of the platelet after stimulation with thrombin was studied by morphologic and immunocytochemical techniques. The use of tannic acid stain and quick-freeze techniques revealed several thrombin-induced morphologic changes. First, the normally discoid platelet became rounder in form, with filopodia, and the granules clustered in its center. The granules then fused with one another and with elements of the surface-connected canalicular system (SCCS) to form large vacuoles in the center of the cell and near the periphery. Neither these vacuoles nor the alpha granules appeared to fuse with the plasma membrane, but the vacuoles were connected to the extracellular space by wide necks, presumably formed by enlargement of the narrow necks connecting the SCCS to the surface of the unstimulated cell. The presence of fibrinogen, β TG, and PF4 in corresponding large intracellular vacuoles and along the platelet plasma membrane after thrombin stimulation was demonstrated by immunocytochemical techniques in saponin-permeabilized and nonpermeabilized platelets. Immunocytochemical labeling of the three proteins on frozen thin sections of thrombin-stimulated platelets confirmed these findings and showed that all three proteins reached the plasma membrane by the same pathway. We conclude that thrombin stimulation of platelets causes at least some of the fibrinogen, β TG, and PF4 stored in their alpha granules to be redistributed to their plasma membranes by way of surface-connected vacuoles formed by fusion of the alpha granules with elements of the SCCS.

Platelets are stimulated by a variety of agents, including thrombin, to change in shape, to release several of the proteins stored in their granules, and to form aggregates. In vivo, these aggregates may form a hemostatic plug. The secretion of the contents of alpha granules from stimulated platelets has received increased attention over the past few years. The release of alpha-granule glycoproteins and proteins is an extremely sensitive marker of platelet activation, since they are released at lower concentrations of stimuli than are necessary for the release of dense granule constituents such as serotonin and ADP. Many granule constituents carry out known functions outside the platelet after release. The levels of platelet-specific proteins in plasma are sensitive indicators of secretion from platelets in vivo and can be useful in the evaluation of certain clinical disorders (1). Biochemical studies have shown that several of the substances secreted from platelets to the extracellular medium (thrombospondin, fibrinogen, fibronectin, Factor VIII-von Willebrand factor, and platelet Factor 4

[PF4])¹ also bind to the platelet plasma membrane, but the functional significance of this binding is unclear (2-7).

The only nonbiochemical technique previously used to study release from platelet alpha granules is immunofluorescence. Although the level of resolution attainable with this technique is too low to permit the differentiation of granule populations, immunofluorescence has been used to localize PF4, fibronectin, Factor VIII-related antigen, and Factor V in platelets. Ginsberg et al. (8) showed that the punctate distribution of PF4 in unstimulated platelets changes to a pattern of large, fluorescent masses upon stimulation of the platelets with thrombin. These intracytoplasmic masses increase in number with time and migrate to the periphery of the cell, but by the time secretion is 90% complete, few masses

¹ Abbreviations used in this paper: β TG, β -thromboglobulin; PF4, platelet Factor 4; SCCS, surface-connected canalicular system.

are observed. These investigators concluded that the intracytoplasmic masses are formed as a result of multiple fusions of granules and may eventually release their contents by fusing with the plasma membrane.

In the present study, we used immunocytochemical methods along with morphologic techniques to investigate the ultrastructural localization of PF4, β -thromboglobulin (β TG), and fibrinogen in permeabilized and nonpermeabilized thrombin-stimulated platelets. Since thrombin stimulates degranulation in the platelet, we also questioned whether release from platelet alpha granules differs from typical exocytosis, that is, fusion of the granule membrane with the plasma membrane such as that observed in pancreas or in polymorphonuclear leukocytes. This report is concerned exclusively with secretion from alpha granules, and does not address the mechanism of secretion from dense bodies or lysosomes. Our findings provide new information on the reorganization of platelet organelles and proteins during degranulation and secretion.

MATERIALS AND METHODS

Washed platelets were prepared at 37°C. Immediately after being drawn, blood was placed in 50-ml polycarbonate tubes with acid-citrate-dextrose anticoagulant and prostaglandin E₁ (Sigma Chemical Co., St. Louis, MO) was added in a final concentration of 5–10 μ g/ml to inhibit platelet activation. The blood was centrifuged for 10 min at 120 g. The platelet-rich-plasma (PRP) was transferred to clean tubes and centrifuged for 10 min at 2,300 g. The resultant pellets were washed twice in 0.14 M NaCl, 0.02 M HEPES, 0.006 M dextrose, 0.001 M EDTA (pH 6.6) using the same centrifugation conditions. Platelets were resuspended in either 0.01 M Tris-HCl, 0.14 M NaCl, 0.006 M dextrose, 0.001 M EDTA (pH 7.4), in Tyrode's buffer with 2 mM CaCl₂, or in Tyrode's buffer with 5 mM EDTA. Either immediately or after being stored for 30 min at 37°C, they were incubated with α -thrombin (gift of John Fenton, New York Department of Health, Albany, NY) at final concentrations of 0.02 to 2.0 U/ml for periods ranging from 5 s to 5 min. Secretion was stopped by the addition of fixative (2% or 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4; 8% paraformaldehyde in 0.1 M PIPES buffer, pH 7.2; or 1.5% glutaraldehyde in 0.1 M Na cacodylate buffer, pH 7.4, 1% sucrose). Platelet samples were fixed for 1 h at 37°C. Some platelet samples were not exposed to thrombin but were fixed immediately after they were stored in resuspension buffer at 37°C.

Tannic Acid Histochemistry: Platelets fixed in glutaraldehyde were incubated with tannic acid (1% tannic acid in 0.05 M Na cacodylate buffer, pH 7.0, no sucrose) for 1 h at room temperature. They were then postfixed in 1% OsO₄ for 1 h, exposed to uranyl acetate for 1 h, dehydrated, and embedded as previously described.

Quick-freeze, Freeze-fracture and Deep-etch, and Freeze-substitution Procedures: Washed platelets were applied to a small piece of Whatman Filter paper #42 (Whatman Laboratory Products, Inc., Clifton, NJ) which was glued to an aluminum specimen platform. A copper block in a quick-freeze apparatus (Polaron Instruments, Inc., Hatfield, PA) was cooled to the temperature of liquid helium by the method of Heuser et al. (9). The platelet suspension was either left unstimulated or injected with 1–5 U/ml of thrombin, and was frozen on the copper block. Quick-frozen platelets were subsequently processed in one of three ways. Some samples were fractured or fractured and deep-etched on a Balzers 360-M freeze-fracture device (Balzers, Hudson, NH) and then shadowed with platinum and carbon. The replicas were cleaned with hypochlorite, washed in distilled water, and mounted on Formvar-coated copper grids. By a modification of the procedure of Hirokawa and Kirino (10), other samples were freeze-substituted by immersion in 5% OsO₄ in acetone for 4 d at –80°C. At the end of this period, they were slowly warmed to room temperature and then incubated for 1 h in 1% uranyl acetate in absolute ethanol. The samples were incubated twice, for 10 min each time, in propylene oxide and then infiltrated, embedded, and prepared for thin sectioning as described above.

Immunocytochemical Procedures: Primary antisera were raised in rabbits against human proteins. Antiserum to β TG was prepared in our laboratory according to published procedures (11). Antiserum to PF4 (12, 13) was affinity-purified and characterized by Dr. Shirley P. Levine. These two antisera were clearly distinguished from one another by immunodiffusion analysis. Antiserum to fibrinogen was affinity-purified by Dr. David R. Phillips.

In control incubations, normal rabbit serum and buffer were used in place of specific primary antiserum.

Platelets were fixed in either 2% or 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), or 8% paraformaldehyde in 0.1 M PIPES buffer (pH 7.2) for 1 h at 37°C. Fixed platelets were washed for 5 min three times in PBS containing 50 mM NH₄Cl. The cells were soaked for 30 min in PBS containing 0.2% (wt/vol) gelatin and 0.005–0.1% (wt/vol) saponin and then incubated at room temperature for 90 min with primary antiserum diluted with the same solution. After incubation with primary antiserum, or control substance, the cells were washed three times over a period of 1 h in the PBS-gelatin-saponin solution, then incubated for 90 min with one of the following reagents, diluted in the wash solution: (a) Fab fragments of sheep anti-rabbit IgG conjugated to peroxidase (Institut Pasteur, Paris, France) in a dilution of 1:50–1:100 (Fab-peroxidase); (b) protein A-peroxidase conjugate (Zymed Laboratories, Burlingame, CA) in a dilution of 1:50–1:100 (pA-peroxidase); or (c) goat anti-rabbit IgG (GAR) linked to colloidal gold (Janssen Pharmaceutics, Beerse, Belgium) in a dilution of 1:10–1:100, depending upon which type of colloidal gold was used (GAR-G5 or GAR-G20). The platelets were washed in PBS three times over 1 h then in 0.1 M Na-cacodylate buffer (pH 7.2). They were fixed at room temperature for 30 min in glutaraldehyde fixative, washed again in Na-cacodylate buffer, and stored overnight at 4°C in the same buffer with 5% sucrose. Cells processed with colloidal gold immunoconjugates were postfixed in 1% OsO₄, exposed to uranyl acetate, and then processed for transmission electron microscopy. Samples processed with immunoperoxidase conjugates were washed several times with 0.1 M Tris-HCl (pH 7.6), preincubated for 10 min with Tris buffer containing 1 mg/ml 3,3-diaminobenzidine, Grade II (Sigma Chemical Co.) and then incubated for 10 min with diaminobenzidine containing 0.01% (vol/vol) H₂O₂. The platelets were washed well with 0.1 M Tris-HCl buffer (pH 7.6) and then with 0.1 M Na-cacodylate buffer, (pH 7.2). They were postfixed in reduced osmium (V. Herzog, personal communication). Briefly, the cells were exposed to 1% unbuffered OsO₄ for 2 min, followed by 15 min in reduced osmium, produced by adding 5 mg potassium ferrocyanide to 2 ml of 1% unbuffered OsO₄. After removal of the reduced osmium the cells were rinsed for 2 min again in 1% unbuffered OsO₄ and dehydrated in a graded series of ethanol, infiltrated with propylene oxide/Epon first in a ratio of 1:1 and then in a ratio of 1:2, and finally infiltrated and embedded in 100% Epon. These samples were not exposed to uranyl acetate. Thin sections were cut, stained with lead citrate, and examined on a Siemens 101 transmission electron microscope.

For studies of the localization of proteins to the plasma membrane, platelets were prepared and processed as described above, except that saponin was omitted from all of the solutions used.

Frozen Thin Section Procedures: Platelets fixed in 8% paraformaldehyde in 0.1 M PIPES buffer (pH 7.2), were washed well in the same buffer containing 10% (wt/vol) sucrose. Platelets fixed in 2% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) were washed well in the same buffer. Both groups were infiltrated for 30 min with 2.3 M sucrose, embedded in the sucrose solution, frozen, and stored in liquid nitrogen. Sections were cut on a Sorvall MT-2 ultramicrotome with a frozen thin section (FTS)/LTC-2 attachment. The frozen thin section techniques described by Tokuyasu (15, 16) were used with the modifications for the use of colloidal gold described by Griffiths et al. (17). The control for these experiments was nonimmune, purified rabbit IgG or buffer in place of the primary antiserum.

RESULTS

The objective of this study was to investigate the distribution of platelet organelles and alpha granule proteins in platelets stimulated with thrombin (0.02–5 U/ml) for various periods (2 s to 5 min) before fixation. These platelets were obtained from anticoagulated blood and washed in the presence of an inhibitor of platelet activation. All procedures through fixation were carried out at 37°C.

Morphological Studies of Thrombin-stimulated Platelets

TANNIC ACID-STAINED PREPARATIONS: To examine platelet degranulation, we used tannic acid to stain the membranes of the platelets in continuity with the external space. This technique also provided better morphologic definition of the plasma membrane than in our immunocyto-

chemical preparations in which the membranes were altered by the saponin used as a permeabilizing agent.

Unstimulated platelets processed with tannic acid had several features well documented by other investigators: a fairly discoid form, and a dispersed arrangement of organelles. Elements of the surface-connected canicular system in these platelets were connected to the plasma membrane by narrow necks and often extended deep into the cytoplasm (Fig. 1*a*). As might be expected, thrombin-stimulated platelets processed with tannic acid had a vastly different appearance. They were round and swollen in form, rather than discoid, and their granules were clustered in the center of the cell, in close apposition with one another and with elements of the surface-connected canicular system (SCCS) (Fig. 1*b*). Occasional images of granule-granule fusion were observed (*inset*, Fig. 1*b*). Some structures were observed in communication with the extracellular space by their dense staining with tannic acid whereas others with a luscious appearance were not. After longer intervals of stimulation with thrombin, large intracellular structures, in continuity with the extracellular space, were present both in the center and at the periphery of the platelet (Fig. 1*c*). The necks joining these elements of the SCCS to the plasma membrane were considerably wider than in unstimulated cells (Fig. 1, *d* and *e*). Granules were sometimes in close proximity to the large intracellular structures, but fusion profiles and pentalaminar formations were rarely observed. We sometimes saw small "dimples" on granules impinging on neighboring granules or elements of the SCCS but we are uncertain of their significance. We more often observed a wide neck between two adjacent structures (Fig. 1*b*, *inset*); through additional fusions, these linked structures may ultimately evolve into large compound granules as seen in Fig. 1*b*.

QUICK-FROZEN PREPARATIONS: We employed the additional technique of quick-freezing in the hope of arresting early events during this extremely rapid degranulation process that may not have been readily observed due to the relatively slow fixation by glutaraldehyde.

In thrombin-stimulated platelets that had been quick-frozen and then either freeze-substituted (Fig. 2*a*), fractured (Fig. 2*b*), or fractured and deep-etched (Fig. 2*c*) we frequently observed large intracellular vacuoles corresponding to those seen in tannic acid-stained preparations.

In unstimulated platelets that had been quick-frozen and then deep-etched, the SCCS sometimes extended into the cytoplasm and was connected to the external surface by narrow necks (Fig. 2*d*). Thrombin-stimulated platelets processed in the same way showed granules in close apposition with one another (Fig. 2*c*, *inset*), and with elements of the SCCS (Fig. 2*e*); the necks connecting the SCCS to the extracellular space were wider than in unstimulated cells.

Demonstration of β TG, PF4, and Fibrinogen in Unstimulated Platelets

FROZEN THIN SECTION EXPERIMENTS: Platelets incubated with antiserum to fibrinogen (Fig. 3, *a* and *b*), PF4 (Fig. 3*c*), or β TG (Fig. 3*d*), and then with GAR-G5 or GAR-G20 showed negligible labeling of the plasma membranes and a similar granule labeling pattern. As in saponin-treated platelets processed with immunoperoxidase conjugates (see next section), not every platelet granule was reactive. Occasionally, label for PF4 was localized to the nucleoid and fibrinogen to

the granule periphery, but this was not a consistent finding. No preferential labeling was observed for β TG. Control samples incubated with nonimmune, purified rabbit IgG (Fig. 3*e*) were almost completely negative.

Demonstration of β TG, PF4, and Fibrinogen in Thrombin-stimulated Platelets

SAPONIN-PERMEABILIZED PLATELETS PROCESSED WITH IMMUNOPEROXIDASE CONJUGATES: To study the intracellular localization of β TG, PF4, and fibrinogen in platelets, we permeabilized unstimulated platelets with saponin and then exposed them to various primary antisera and Fab-peroxidase.

In unstimulated platelets processed with antifibrinogen (Fig. 4*a*), anti- β TG, or anti-PF4 serum, reaction product was localized in the alpha granules, and was negligible on the plasma membrane. To investigate the redistribution of these substances after thrombin stimulation, we processed thrombin-stimulated platelets in the same way. The results with antibodies against fibrinogen (Fig. 4, *b-d*), β TG, and PF4 were the same. Large intracellular vacuoles filled with reaction product were sometimes present in the center of the cell, together with a few reactive or unreactive granules. Pools of reaction product were frequently present near the periphery of the platelet (Fig. 4, *b-d*), but we saw no images of direct fusion with the plasma membrane. The plasma membrane was devoid of reaction product probably because of the saponin treatment. No reaction product was visible on the plasma membranes or in intracellular vacuoles in control preparations incubated with normal rabbit serum rather than the specific primary antiserum (Fig. 4*e*).

It should be noted that morphologic preservation was compromised because of the saponin treatment necessary to allow access of reagents. In addition, we frequently observed "bleeding" of peroxidase reaction product into the platelet cytoplasm, as a result of both weak fixation and saponin treatment. Increasing the concentration of paraformaldehyde in the fixative to 8% yielded better morphologic detail, but fewer reactive granules, although the reactive granules were intensely stained.

Localization of β TG, PF4, and Fibrinogen on the Plasma Membranes of Nonpermeabilized Platelets

Because biochemical studies have demonstrated that some of the substances secreted from platelet granules bind to the platelet plasma membrane (2-7, 18), we examined the extracellular localization of β TG, PF4, and fibrinogen after thrombin stimulation in nonsaponin-treated platelets prepared in resuspension buffer containing 0.001 M EDTA.

PLATELETS PROCESSED WITH IMMUNOPEROXIDASE CONJUGATE: Because we previously found that Fab-peroxidase bound nonspecifically to the plasma membrane, we used pA-peroxidase to study the localization of β TG, PF4, and fibrinogen on the plasma membranes of unstimulated and thrombin-stimulated platelets. In samples incubated with antifibrinogen serum and then pA-peroxidase, the plasma membranes of unstimulated platelets were almost free of reaction product (Fig. 5, *a*), whereas the plasma membranes of thrombin-stimulated cells were densely labeled (Fig. 5, *b*). This was also true for PF4 and β TG, although the labeling

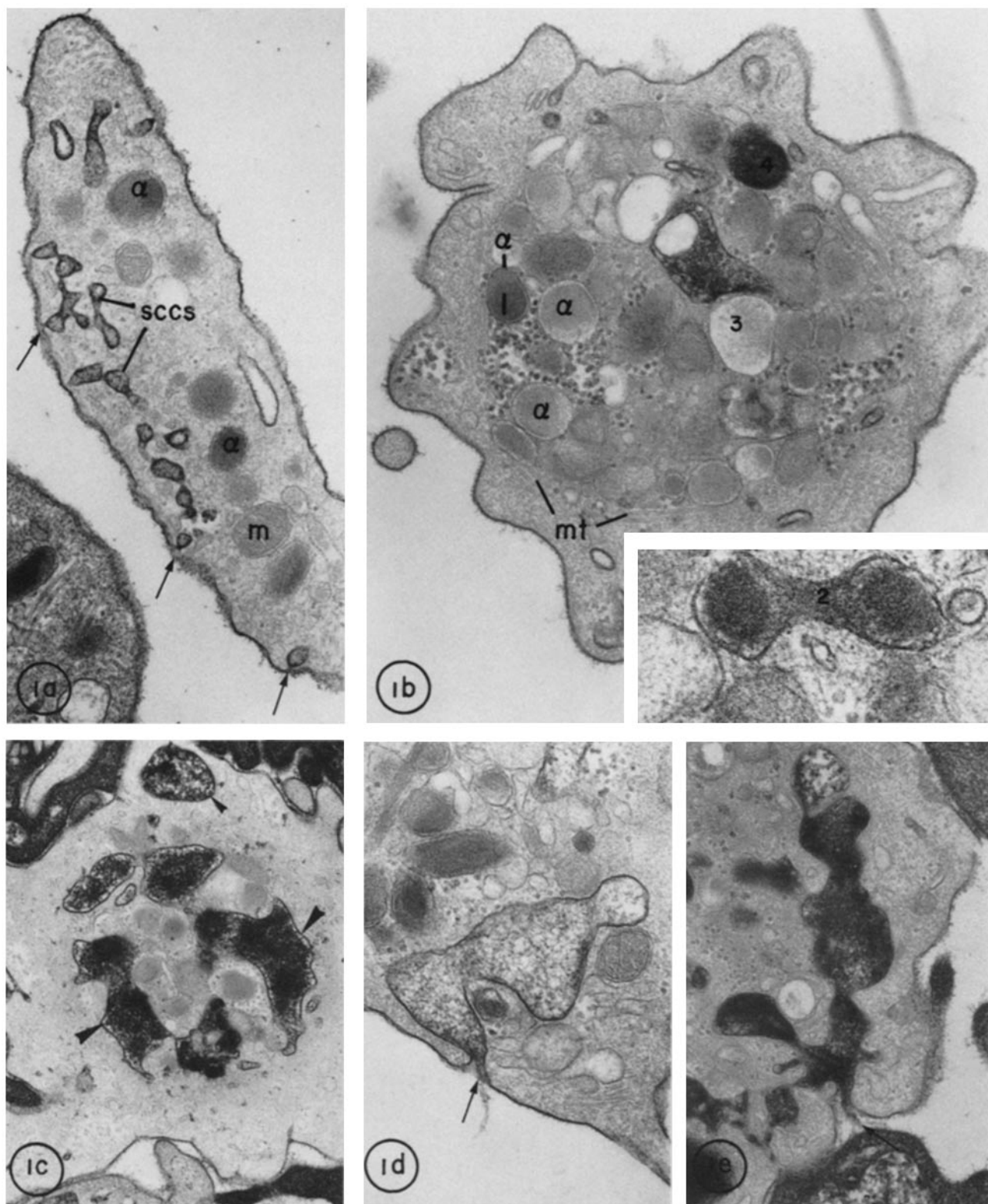


FIGURE 1 Transmission electron micrographs of platelets processed with tannic acid stain to elucidate platelet degranulation after short intervals of thrombin stimulation. Tannic acid densely stains the membranes of the platelet in continuity with the extracellular space. (a) Platelet from an unstimulated sample. Alpha granules (α) and mitochondria (m) are dispersed throughout the cytoplasm. The SCCS, densely stained by tannic acid, is connected to the plasma membrane by narrow necks (arrows). (b–d) Platelets from samples stimulated with 1 U/ml of thrombin for various intervals (5–30 s) before fixation. (b). Alpha granules (1) are clustered in the center of the cell and are surrounded by a ring of microtubules (mt). Some of the granules appear to be fusing (2, inset). The fused granules form large intracellular vacuoles of varying densities, some of which are not yet in contact with the external space (3), and some of which are (4). (c) Large structures containing tannic acid are present in the cytoplasm, both in the center of the cell (large arrowheads) and near the periphery (small arrowhead). (d and e) The large tannic acid-filled structures are in continuity with the extracellular space. The necks connecting these elements of the SCCS to the extracellular space (arrows) are considerably wider than those in a. All samples were fixed in glutaraldehyde, stained with tannic acid, and postfixed in OsO_4 . Sections were stained on grid with uranyl acetate and alkaline lead citrate. (a, b, d, and e) $\times 34,000$; (b, inset) $\times 96,000$; (c) $\times 21,000$.

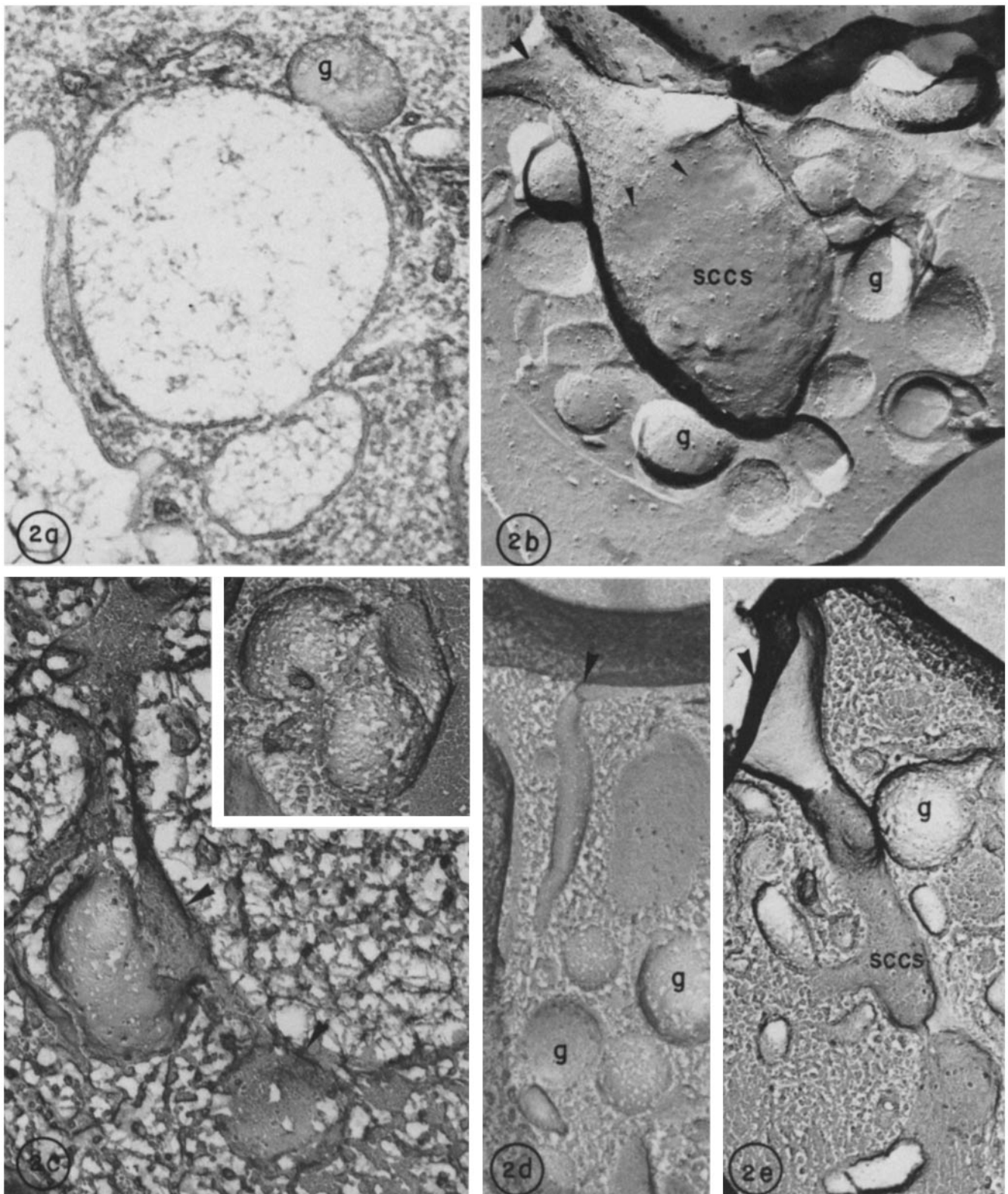


FIGURE 2 Transmission electron micrographs of thrombin-stimulated, quick-frozen platelets. All samples but that shown in *d* were exposed to 1–5 U/ml of thrombin for 2 s before being frozen. (a) Freeze-substituted sample. A granule (*g*) is fusing with a large vacuolar structure, which corresponds to the structure labeled 3 in Fig. 1*b*. (b) Fractured sample. Granules are clustered around the large SCCS, which opens to the external space (large arrowhead). Two areas cleared of intramembranous particles on the E face of the SCCS (small arrows) may represent granules that have come into close apposition with the SCCS preparatory to fusing with this structure. (c–e). Fractured and deep-etched samples. Two granules (arrowheads) are in continuity with a large vacuolar structure. (d) Unstimulated sample. The SCCS forms an invagination into the cytoplasm from a narrow opening on the plasma membrane (arrowhead), corresponding to the openings seen in Fig. 1*a*. (e) A granule (*g*) is in close apposition with the SCCS, which extends deep into the cytoplasm and which is joined to the plasma membrane by a wide neck (arrow), corresponding to the necks seen in Fig. 1, *d* and *e*. *g*, granule; SCCS, surface-connected canicular system. (a–c) $\times 80,000$; (d and e) $\times 62,000$; (inset, c) $\times 85,000$.

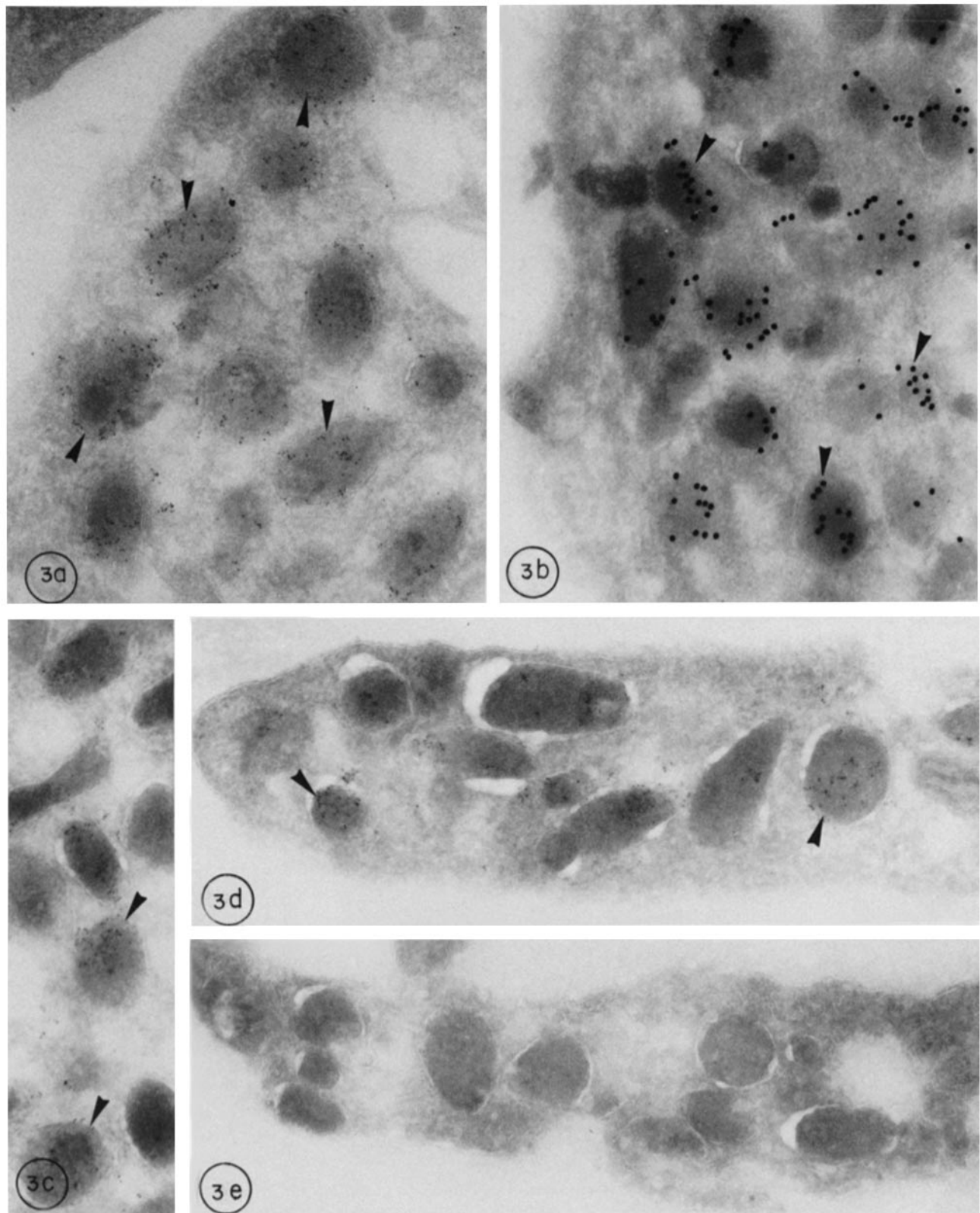


FIGURE 3 (a-e) Transmission electron micrographs showing the localization of proteins in washed platelets fixed in 8% paraformaldehyde and prepared for frozen thin section immunocytochemistry. (a and b) Sections incubated with antifibrinogen serum and then with GAR-G5 (a) and GAR-G20 (b). (c) Sections incubated with anti-PF4 serum and then GAR-G5. (d) Sections incubated with anti- β TG serum and then GAR-G5. Note the heavy labeling (arrowheads) of most of the granules and the paucity of label on the plasma membrane. (e) Sections incubated with nonimmune, purified rabbit IgG and then GAR-G5. Note that the section is free of label. $\times 50,000$.

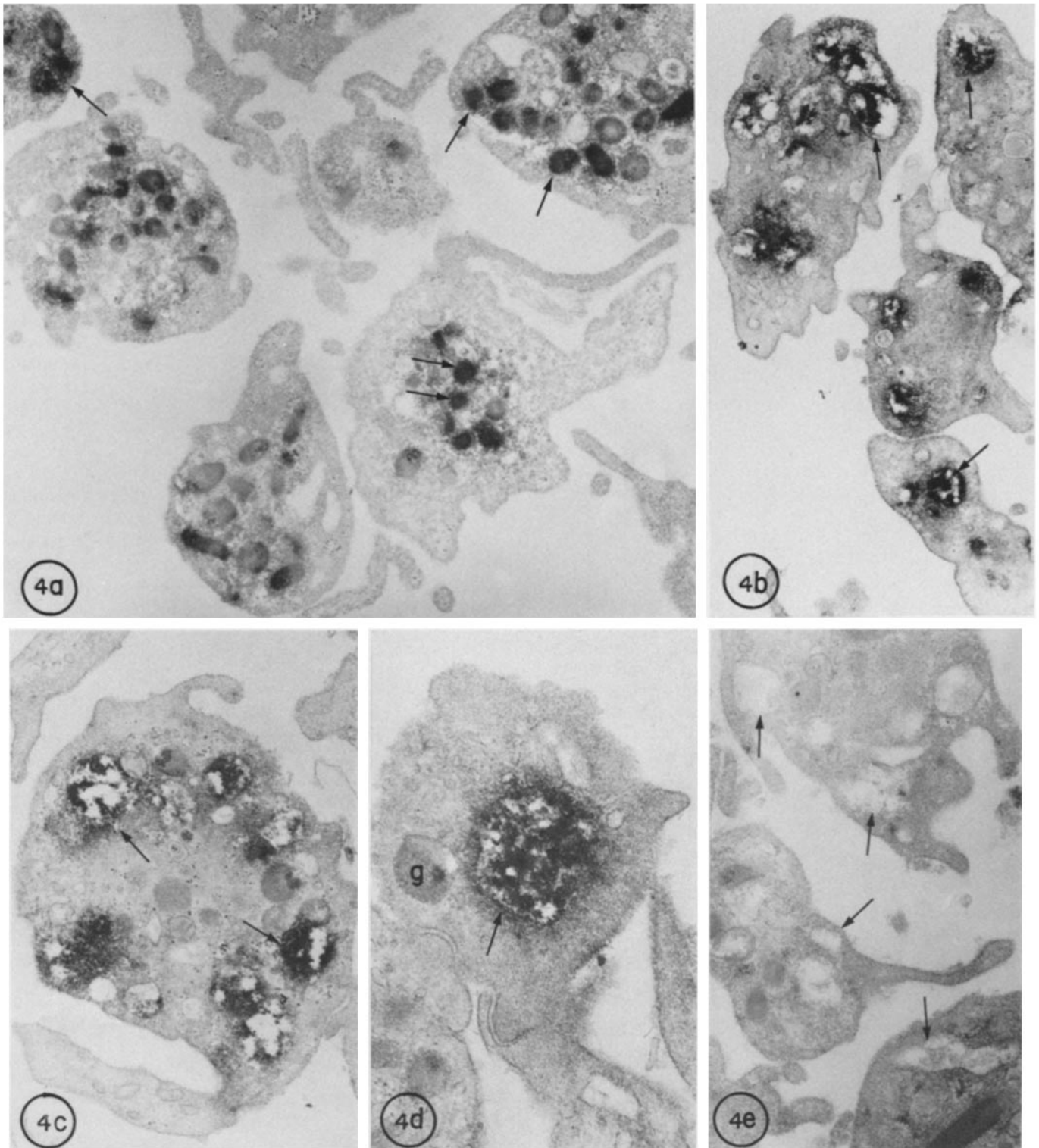


FIGURE 4 Transmission electron micrographs showing the localization of fibrinogen in fixed, permeabilized platelets before (a) and after (b-d) thrombin stimulation to illustrate the redistribution of alpha-granule contents. (a-d) Washed platelets incubated with antifibrinogen serum and then Fab-peroxidase. (a) Unstimulated platelets. Note the dense reaction product uniformly filling many of the alpha granules (arrowheads). Some of the granules are less reactive than others, and some are negative. (b-d) Platelets incubated with thrombin (0.2-1 U/ml) for various periods (5-30 s) before fixation. Note the heavily reactive intracellular structures in the cytoplasm (arrowheads), both near the centers of the cells and near the peripheries. Some of the granules (g) are not reactive. Note that the plasma membrane is not labeled. (e) Control preparation processed as in b-d, except that normal rabbit serum was used in place of antifibrinogen serum. The intracellular vacuoles (arrows) do not contain reaction product. Unstimulated platelets were fixed in 2% paraformaldehyde and permeabilized with 0.05% saponin; thrombin-stimulated platelets were fixed in 4% paraformaldehyde and permeabilized with 0.1% saponin. (a and b) $\times 16,000$; (c) $\times 19,500$; (d) $\times 29,000$; (e) $\times 21,000$.

was less dense for β TG than for fibrinogen or PF4. In control samples incubated with normal rabbit serum and then pA-peroxidase after fixation, the plasma membranes were free of label (Fig. 5, c).

PLATELETS PROCESSED WITH COLLOIDAL GOLD IMMUNOCONJUGATES: Unstimulated platelets incubated with antifibrinogen serum were free of IgG-gold label (Fig. 5, d), whereas thrombin-stimulated platelets incubated with the same antisera had discrete clumps of IgG-gold label around the entire plasma membrane (Fig. 5, e and f). As in platelets processed with immunoperoxidase conjugate, the labeling was frequently heaviest where platelets were closely apposed. The pattern of labeling was the same for β TG and PF4 although the labeling was least dense for β TG.

Morphologic Demonstration of the Ca^{++} -dependent Binding of Secreted Fibrinogen to the Platelet Plasma Membrane

Previously it was shown by biochemical techniques that several alpha granule proteins bind to platelets in the presence of Ca^{++} (7, 19). We therefore examined at the fine structural level the binding of fibrinogen to the platelet plasma membrane in buffer containing either 2 mM CaCl_2 or 5 mM EDTA.

PLATELETS PROCESSED WITH COLLOIDAL GOLD IMMUNOCONJUGATES: Thrombin-stimulated platelets prepared in Tyrode's buffer with 2 mM CaCl_2 had heavy label for fibrinogen (Fig. 6a) along the platelet plasma membranes at the peripheries of aggregates. Little label was seen in the central portions of aggregates. Little or no label was observed for fibrinogen on platelets stimulated with thrombin in Tyrode's buffer with 5 mM EDTA (Fig. 6b). These results were the same for PF4 and β TG, although considerably less label was seen for β TG.

FROZEN THIN SECTIONS PROCESSED WITH COLLOIDAL GOLD IMMUNOCONJUGATES: The use of colloidal gold immunoconjugates on frozen thin sections allowed us to examine the intracellular and extracellular distribution of proteins secreted from alpha granules. Furthermore, it permitted us to circumvent the permeation and "bleeding" problems in saponin-treated preparations and to use a different second marker. In thrombin-stimulated platelets prepared in Tyrode's buffer with 2 mM CaCl_2 , fibrinogen (Fig. 6c), β TG (Fig. 7a), and PF4 (Fig. 7b) were localized along the plasma membranes of aggregated platelets and throughout the aggregates, in large intracellular vacuoles and in masses of granules. The least amount of label was seen for β TG. A significant

decrease in label along the platelet plasma membranes for fibrinogen (Fig. 6d), β TG (not shown), and PF4 (not shown) was observed when platelets were stimulated in Tyrode's buffer with 5 mM EDTA. Large intracellular vacuoles and some residual granules were heavily labeled, however.

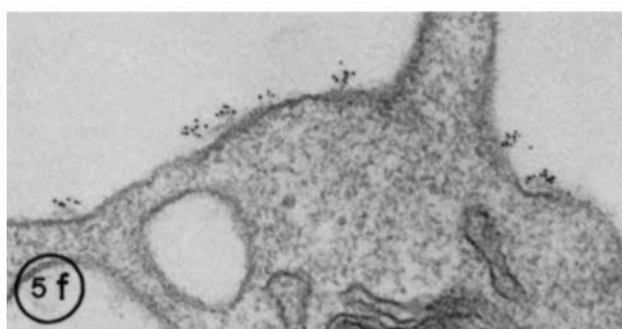
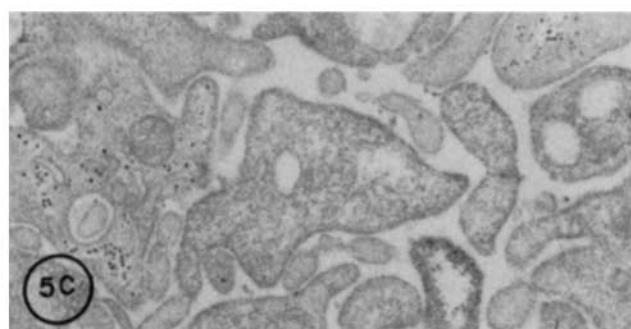
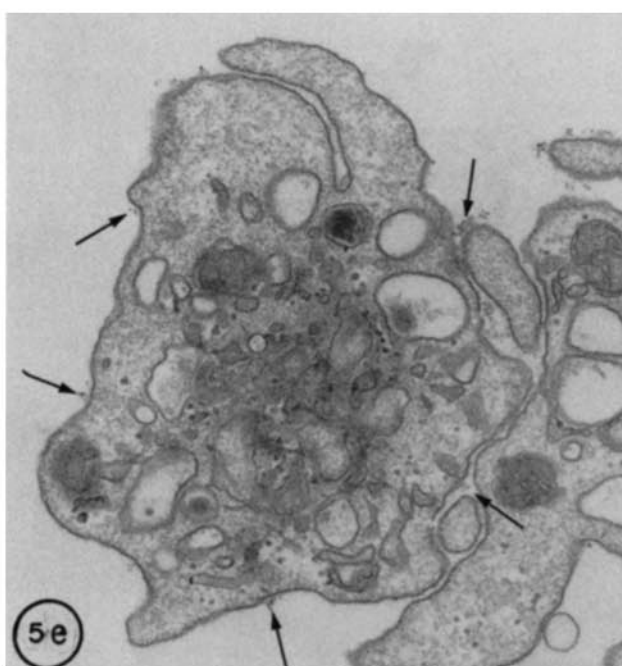
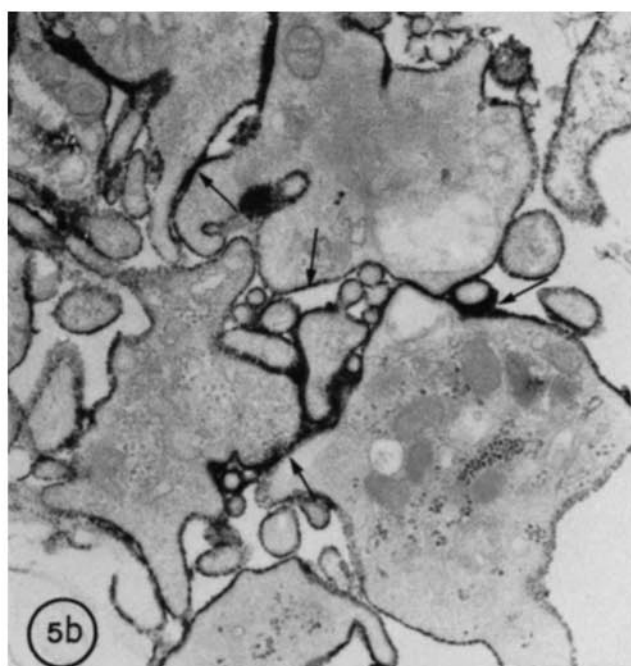
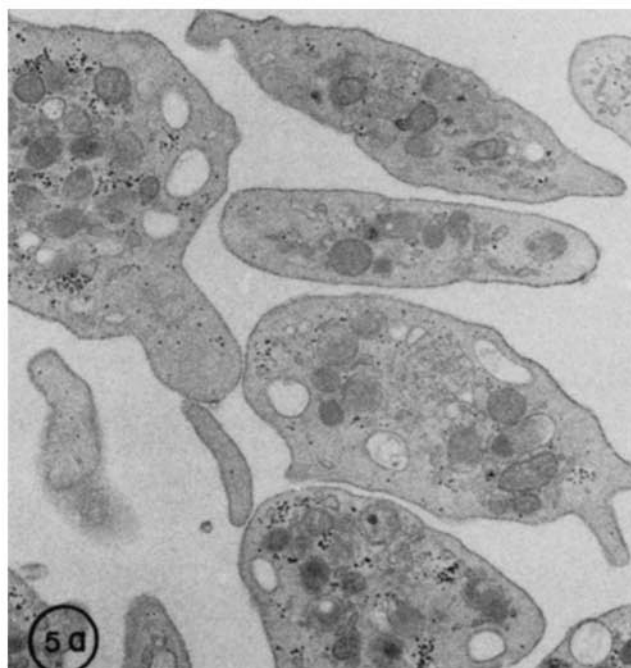
Thus, our morphological results confirm the previous biochemical demonstration of Ca^{++} -dependent binding of fibrinogen and PF4 to platelets. Binding of β TG to platelets has not been previously observed. Although we found much less β TG associated with the platelet surface than fibrinogen or PF4, this binding appeared to be specific in that it was Ca^{++} -dependent.

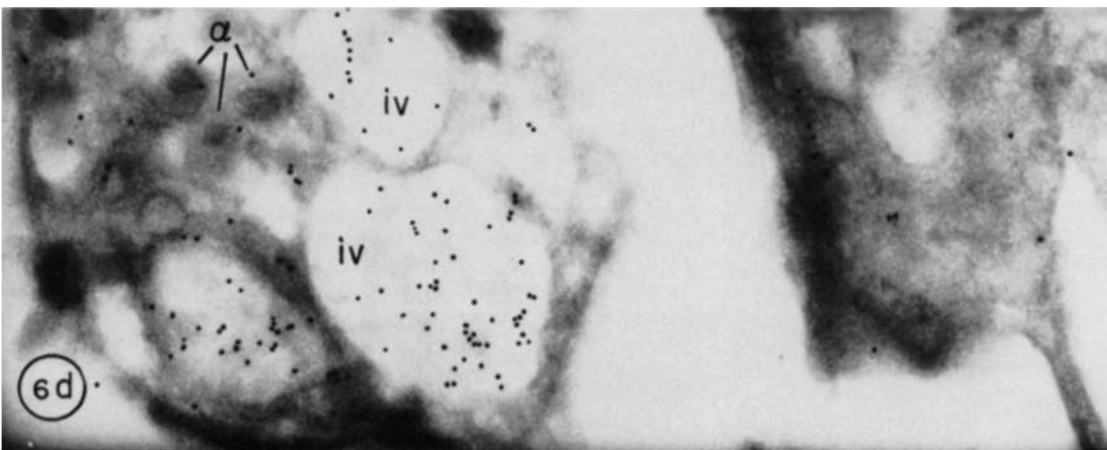
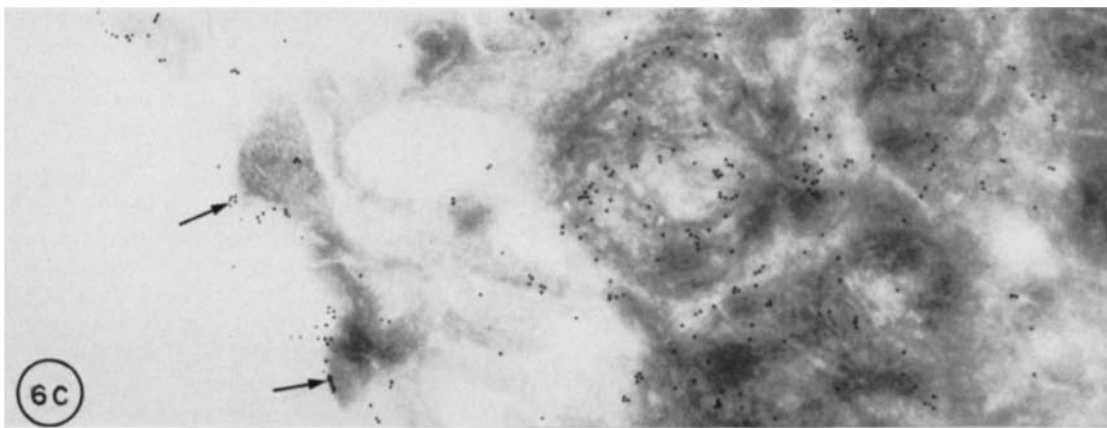
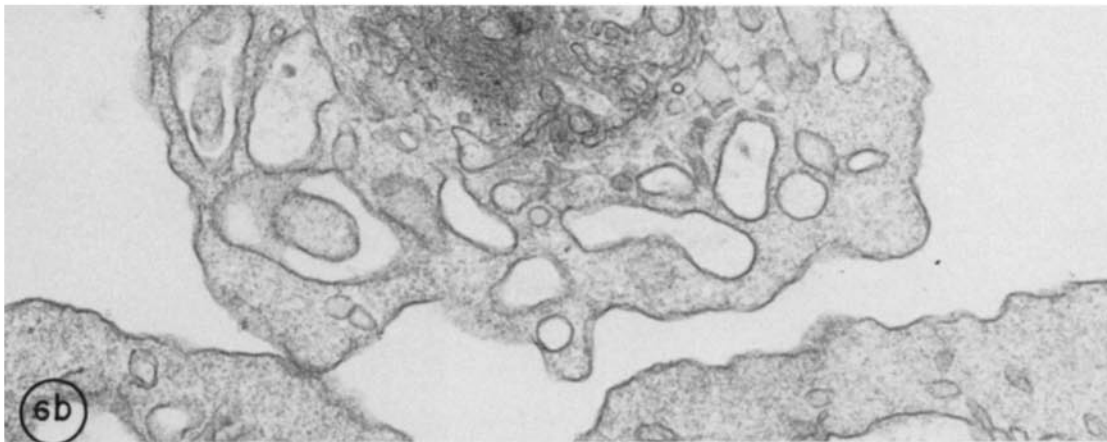
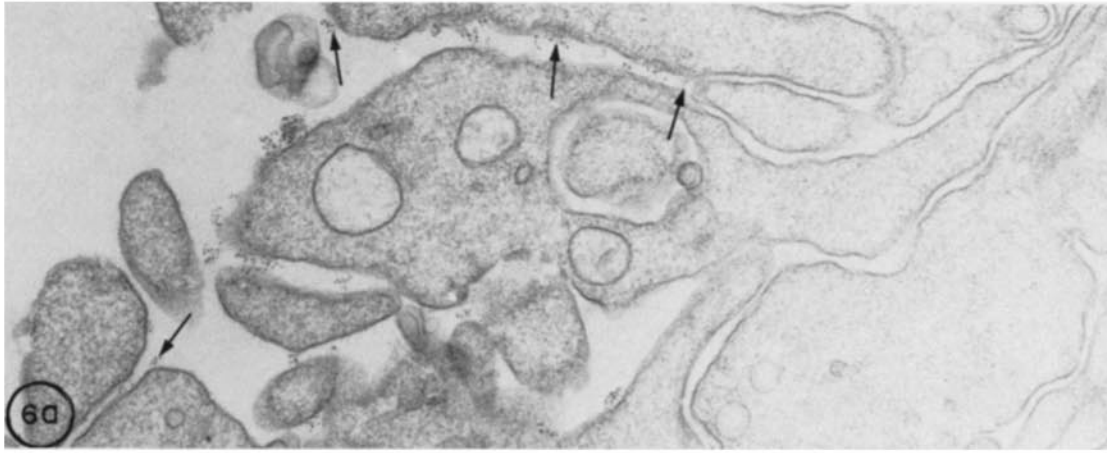
DISCUSSION

In the present investigation, we have traced the pathway by which β -thromboglobulin, platelet Factor 4, and fibrinogen are secreted from the platelet after stimulation with thrombin. Staining with tannic acid and quick-freeze techniques revealed that thrombin stimulation resulted in various morphologic changes in the platelet, including fusion of the alpha granules with one another and with elements of the SCCS to form large, surface-connected structures. Immunocytochemical labeling of β TG, PF4, and fibrinogen showed that thrombin stimulation caused them to be redistributed from the alpha granules to large intracytoplasmic vacuoles and to the platelet plasma membrane. These vacuoles ultimately fused with the SCCS and were connected to the extracellular space by wide necks, presumably formed by enlargement of the narrow necks connecting the SCCS to the surface of the unstimulated cell. Since fusion of the alpha granules or the large vacuoles with the plasma membrane was rarely seen using these techniques, we conclude that these substances gained access to the external milieu through these wide necks.

These morphologic and immunocytochemical observations suggest the following pattern of secretion from the platelet alpha granules after thrombin stimulation. The normally discoid platelet first becomes round and filopodia develop after stimulation with thrombin. Concomitantly, the alpha granules and other organelles move to the center of the platelet and are surrounded by a ring of microtubules. Granules fuse with other granules and with elements of the SCCS to form large vacuoles, situated near the center and periphery of the platelet, and containing alpha-granule proteins. As these vacuoles form in the cytoplasm, the necks connecting the SCCS to the extracellular space become wider. The enlargement of these openings in the plasma membrane permits the rapid secretion of alpha granule proteins to the exterior of the

FIGURE 5 Transmission electron micrographs of unstimulated and thrombin-stimulated platelets prepared in resuspension buffer with 0.001 M EDTA, processed without saponin, and examined immunocytochemically for the extracellular distribution of fibrinogen. (a) Unstimulated platelets exposed to antifibrinogen serum and then pA-peroxidase. Note that the membranes of these cells are relatively free of peroxidase label. (b) Platelet aggregate from a thrombin-stimulated sample incubated with antifibrinogen serum and pA-peroxidase. The dense reaction product that is present along most of the platelet plasma membranes (arrows) is particularly heavy where two platelets are in close apposition. None of the granules are reactive because the cells have not been permeabilized. (c) Control preparation. Part of a platelet aggregate from the same sample as b. Cells were incubated with normal rabbit serum and then pA-peroxidase. The plasma membranes are free of peroxidase label. (d) Unstimulated platelets exposed to antifibrinogen serum and then GAR-G5. The plasma membranes are free of gold label. (e) Platelet aggregate from the same sample as b. Cells were incubated with antifibrinogen serum and then GAR-G5. The plasma membrane is labeled in a patchy manner along its entire extent (arrows). (f) Higher magnification of the sample shown in e. The gold particles are distributed in discrete groups along the extent of the platelet plasma membrane. Unstimulated platelets and thrombin-stimulated platelets (1 U/ml for 5 min) were fixed in 2% paraformaldehyde. (a and b) 17,300; (c) $\times 21,800$; (d and e) $\times 30,000$; (f) $\times 72,000$.





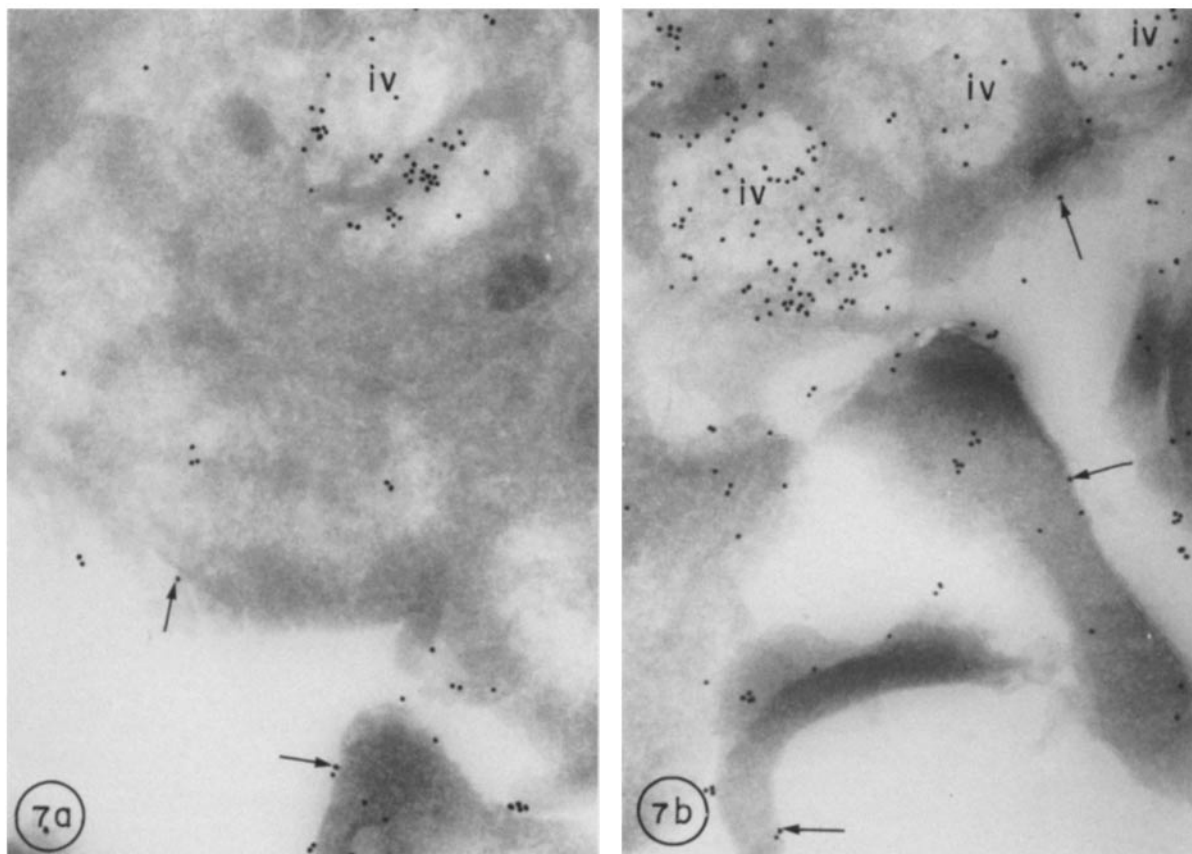


FIGURE 7 Transmission electron micrographs of thrombin-stimulated platelets prepared in Tyrode's buffer with 2 mM CaCl_2 and processed for frozen thin section immunocytochemistry to examine the distribution of βTG (a) and PF4 (b). (a) Sections were exposed to anti- βTG serum and then GAR-G20. Note that there is label along the plasma membranes of aggregated platelets (arrows) as well as heavy label within an intracellular vacuole (iv). (b) Sections were exposed to anti-PF4 serum and then GAR-G20. Note the heavy label along the plasma membranes of aggregated platelets (arrows) as well as in large intracellular vacuoles (iv). In general the label on the plasma membrane was less for βTG (a) than PF4 (b), or fibrinogen (see Fig. 6c). Thrombin-stimulated platelets (1 U/ml for 5 min) were fixed in 8% paraformaldehyde. (a) $\times 34,500$; (b) $\times 37,500$.

platelet and binding of some of this secreted protein to the plasma membrane, where they carry out their various physiological functions.

This summary is consistent with other ultrastructural findings on platelet degranulation. The localization of various electron dense tracers (lanthanum nitrate, ruthenium red, horseradish peroxidase, thorium dioxide) in stimulated platelets suggested to White that the SCCS was a probable pathway for release of platelet products (20–23). Studies of uptake and release of polylysine and polybrene by platelet granules (24)

as well as studies of platelets treated with phorbol myristate acetate (25) and cytochalasin B and trypsin (26) led him to conclude that degranulation occurred through fusion of the secretory organelles with elements of the SCCS. Our data supports this hypothesis. In studies of thrombin-stimulated platelets, Droller (27, 28) postulated that an electron-dense, fibrous material observed within an intracytoplasmic "lamellar-vacuolar network" was fibrinogen that had been secreted from the alpha granules. Holme et al. (29) proposed that fibrils observed in the SCCS of thrombin-stimulated platelets

FIGURE 6 Transmission electron micrographs of thrombin-stimulated platelets prepared in Tyrode's buffer with either 2 mM CaCl_2 (a and c) or 5 mM EDTA (b and d) and examined immunocytochemically for the distribution of fibrinogen. (a) Platelets prepared in Tyrode's buffer with CaCl_2 were exposed in suspension to antifibrinogen serum and then GAR-G5. Note the heavy label along the plasma membranes of platelets at the periphery of the aggregate (arrows) and the decreased amount of label further into the mass of cells (right hand). This illustrates a technical problem associated with formation of large aggregates after thrombin stimulation, one of penetration of antibodies into the mass of cells. (b) Platelets prepared in Tyrode's buffer with EDTA processed identically to a. The plasma membranes are relatively free of gold label compared with a. (c) Platelets prepared in Tyrode's buffer with CaCl_2 processed for frozen thin section immunocytochemistry and the sections exposed to antifibrinogen serum and then GAR-G20. Note the heavy label along the plasma membranes of aggregated platelets (arrows) as well as in the center of the mass of cells. (d) Sections of stimulated platelets prepared in Tyrode's buffer with EDTA processed identically to c. The plasma membranes of these cells are relatively free of gold label, although large intracellular vacuoles (iv) and some remaining granules (α) are labeled. Thrombin-stimulated platelets (1 U/ml for 5 min) were fixed in either 2% paraformaldehyde (for suspension experiments) or 8% paraformaldehyde (for frozen thin section experiments). (a and b) $\times 36,000$; (c) $\times 22,000$; (d) $\times 32,000$.

originated from fibrinogen, because they were not present when the platelets had been incubated with iodoacetic acid and antimycin before stimulation. These studies support our findings that platelet fibrinogen is secreted to the extracellular space via the SCCS. Zucker-Franklin (30) has demonstrated by freeze-fracture and electron microscopic techniques two pathways for platelet endocytosis: membrane pits, which endocytose small particles and solutes; and membrane invaginations, which endocytose large particles. She has speculated that these pathways may also be involved in secretion. The membrane invaginations may correspond to distended elements of the SCCS. Finally, using immunofluorescence techniques on detergent-permeabilized platelets, Ginsberg and colleagues (8) showed that PF4 had a granular distribution in the resting cell, but was distributed in large masses near the periphery of the cell after thrombin stimulation. Although these observations are consistent with a redistribution of PF4 from the alpha granules to large vacuoles, these investigators have suggested that such vacuoles are membrane-bounded structures that either are in a canalicular system which is functionally closed at times (8) or that release their contents by fusing with the plasma membrane (8, 31). The data presented in this paper supports the former interpretation of their data.

Our finding that at least some β TG, PF4, and fibrinogen bind to the surface of the platelet after thrombin-induced secretion from the cell is supported by biochemical studies (2–7, 18) showing such binding of thrombospondin, fibrinogen, fibronectin, Factor VIII-von Willebrand Factor, Factor V, and PF4. Other biochemical studies have shown that stimulated platelet membranes bear specific receptors for fibrinogen, fibronectin, and Factor VIII-related antigen (32–35) and that membrane-specific binding of platelet Factor 4 and fibrinogen appears to be Ca^{++} -dependent (7, 19). The binding of secreted fibrinogen, β TG, and PF4 in the presence of Ca^{++} to the plasma membrane observed by both frozen thin section immunocytochemistry and immunocytochemistry on nonpermeabilized cells in suspension is probably specific, since little label was observed in preparations that included EDTA. We would like to point out, however, that there was considerably less label when β TG antiserum was used, confirmatory of our earlier studies (36). Whether the various alpha-granule proteins bind to the plasma membrane in different proportions after secretion is not known. For example, the characteristics of β TG binding to the platelet plasma membrane have not yet been determined. It is possible that only a small portion of the total secreted protein binds to the plasma membrane. George and Onofre (7) suggest that the binding, which localizes and concentrates the secreted substances on the platelet surface, may facilitate platelet functions such as adhesion and aggregation.

We have traced the pathway by which three proteins are secreted from the alpha granules of the platelet after thrombin stimulation. Whether other alpha granule proteins are secreted by this pathway, and whether stimuli other than thrombin induce secretion by this pathway, remain to be determined. It is also unclear whether the other platelet granule populations, dense bodies, lysosomes, and peroxisomes, release their contents through a similar pathway, or indeed, if peroxisomes release their contents at all. The kinetics of secretion vary from one granule population to another. Dense body and alpha granule constituents appear to be released at the same time, while lysosomal contents are released after

alpha granule and dense body secretion. Alpha granule proteins are secreted at lower concentrations of stimuli such as thrombin than are dense body and lysosomal constituents (37–39). Lastly, thrombin-stimulated platelets release different amounts of the various acid hydrolases stored in their lysosomes: equal amounts of β -N-acetylglucosaminidase, β -galactosidase, and cathepsin; smaller, but equal, amounts of β -glucuronidase and aryl sulfatase; and no acid phosphatase (40). Holmsen and Day (41) have suggested that these substances may be contained in different granules or that some proteins may be bound more strongly in the same granule than others, and therefore not released to the same extent. It is clear that more work is needed to determine the secretory pathways of these other granule populations.

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Note added in proof: Recently, Sander *et al.* reported similar distributions of these proteins in unstimulated platelets by immunocytochemistry on frozen thin sections (*J. Clin. Invest.* 72:1277a, 1983). In addition, the paper entitled "Optimal techniques for the immunocytochemical demonstration of β -thromboglobulin, platelet Factor 4, and fibrinogen in alpha granules of unstimulated platelets" is in press in *The Histochemical Journal*, 1984.

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